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INITIAL SUB- MISSION

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RE: Benzene, 1,1'-methylenebis[isocyanato-
Chemical Abstracts Service Number 26447-40-5

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Dear Sir/Madam,

The following information is being submitted by the International Isocyanate Institute, Inc.* on behalf of its members pursuant to current guidance by EPA indicating EPA's interpretation of section 8(c) of the Toxic Substances Control Act. Neither IRI nor any member of IRI has made a determination as to whether a significant risk of injury to health or the environment is actually presented by the findings.

Chemical substance: benzene, 1, 1'-methylenebis[isocyanato-
CAS #: 26447-40-5

Study Title: ELISA development and analysis of rat anti-MDI IgG antibody responses following dermal MDI exposure

Author: R. Dearman of Central Toxicology Laboratory, Macclesfield, Cheshire, UK

IRI ref #: 11420

The report by R. Dearman describes the assay of anti-MDI IgG antibody in sera of rats exposed by dermal or intra-dermal route to MDI. Responses have been previously reported in 1994 from Dearman's laboratory by Rattray et al. ("Induction of Respiratory Hypersensitivity to Diphenylmethane-4,4'-diisocyanate (MDI) in Guinea Pigs. Influence of Route of Exposure",

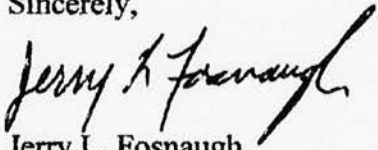
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Toxicology, Vol. 88, Nos. 1-3, pages 15-30). Studies by Pauluhn indicate that an immediate onset respiratory hypersensitivity response could only be elicited from intra-dermally sensitized animals with concentrations exceeding the irritant threshold concentration for MDI (Pauluhn, J. and Mohr, U., Toxicology; Vol 92, ISS 1-3, 1994, P53-74, "Assessment of respiratory hypersensitivity in guinea-pigs sensitized to diphenylmethane-4,4'-diisocyanate (MDI) and challenged with MDI, acetylcholine or MDI-albumin conjugate").

The significance of antibody response has been questioned, even in humans. A recent publication describing a NIOSH investigation concluded "serum concentrations of MDI specific IgG appear to be a moderately sensitive biological marker of MDI exposure, but not an indicator of occupational asthma" (Lushniak BD, et al. American Journal of Industrial Medicine, Vol. 33, No. 5, pages 471-477, "Indirect Assessment of 4,4'-Diphenylmethane Diisocyanate (MDI) Exposure by Evaluation of Specific Humoral Immune Responses to MDI Conjugated to Human Serum Albumin").

Sincerely,



Jerry L. Fosnaugh
Managing Director Designee

*"The International Isocyanate Institute, Inc. members are producers of MDI and TDI. In the U.S., the members are Huntsman Polyurethanes, The Dow Chemical Company, BASF Corp., Bayer Corp. and Lyondell Chemical Co."

III Project 126

III ref. 11420

ELISA Development and analysis of rat anti-MDI IgG antibody responses following dermal MDI exposure

R Dearman

**Central Toxicology Laboratory
Macclesfield
Cheshire, UK**

April 2001

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III Report

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**CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
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REPORT NO: CTL/L/8846

**ELISA DEVELOPMENT AND ANALYSIS OF RAT
ANTI-MDI IgG ANTIBODY RESPONSES**

**CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
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REPORT NO : CTL/L/ 8846

**ELISA DEVELOPMENT AND ANALYSIS OF RAT
ANTI-MDI IgG ANTIBODY RESPONSES**

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Summary

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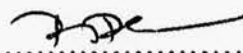
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DATE OF ISSUE

STATEMENT OF COMPLIANCE

This report is based on carefully conducted research studies which, however, are not fully compliant with Good Laboratory Practice (GLP) standards. The data described in this summary report have not been audited by the Central Toxicology Laboratory (CTL) Quality Assurance Unit.

R J Dearman
Study Investigator

 14/2001

P M Hext
Product Manager,
Central Toxicology Laboratory

 18/4/2001

I, the undersigned, declare that this report constitutes a true record of the actions taken and the results obtained in the above study.

R J Dearman
Study Investigator


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Date 17/4/2001.....

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1. SUMMARY

An enzyme-linked immunosorbant assay (ELISA) was developed for the detection of anti-MDI IgG antibody in rat serum using a conjugate prepared with MDI monomer and human serum albumin (HSA). Serum isolated from rats injected intradermally or exposed topically to MDI displayed detectable anti-MDI IgG antibody with similar kinetic profiles and magnitude of responses. The kinetics and the magnitude of the antibody response to topically applied MDI were unaffected by the introduction of a skin wash 8 hours after topical treatment, indicating that the majority of the immunogenically reactive MDI was delivered within 8 hours of exposure. The fact that extended duration of exposure does not appear to increase the immune response may be attributable also to a short biological half-life of this molecule in biological fluid. Antibody responses observed following intradermal or topical application of MDI were of relatively low titre. It should be noted however that dose-responses were not performed in these experiments, making interpretation of the relationship between topical and intradermal exposure difficult.

Thus in the rat both intradermal injection and topical exposure to MDI induces significant but relatively low levels of anti-MDI IgG antibody. However, it is not possible to determine from these experiments whether the route of exposure is the important variable or whether this strain of rat is inherently less responsive to MDI regardless of the route of exposure employed.

2. INTRODUCTION

As an amendment to III project 126-EU-MTX (Dermal penetration study with ^{14}C -MDI in rats), additional animals were exposed dermally to MDI, with and without a skin wash at 8 hours, and serum samples were prepared at various times after exposure. Additional positive control rats received a single intradermal injection of MDI. The serum samples were received from BASF, Department of Toxicology, Ludwigshafen, Germany on May 5th 1999.

The agreement with the International Isocyanates Institute was that an enzyme-linked immunosorbent assay (ELISA) would be developed for the detection of anti-MDI specific

IgG antibody in rat sera using pooled serum samples from the positive control animals exposed intradermally to MDI. Individual serum samples from the animals exposed dermally to MDI would then be analyzed for anti-MDI IgG antibody content using this ELISA.

3. MATERIALS AND METHODS

3.1 Preparation of conjugate

Monomeric MDI was provided by Bayer AG. The material as supplied as 99.6% diphenylmethane-4-4'-diisocyanate, 0.37% diphenylmethane-2,4-diisocyanate and less than 5ppm phenyl isocyanate and was stored at 4°C under nitrogen. MDI-rat serum albumin (RSA, Fraction V; Sigma Chemical Company, St Louis, MO), MDI-human serum albumin (HSA, Fraction V; Sigma) or guinea pig serum albumin (GSA, Fraction V; Sigma) conjugates were prepared. Approximately 200mg of RSA, HSA or GSA was dissolved in 20 ml of phosphate buffered saline (PBS; pH 7.2) and the material buffered to pH 7.0 using 0.1M NaOH. Approximately 60mg of monomeric MDI was added and the solution stirred at room temperature for 3hr. Glass vessels were used throughout as MDI reacts with plastic. The solution was passed through a G25 Sephadex column to remove any unbound MDI and dialyzed successively against PBS and distilled water for a period of approximately 48hr at room temperature. The conjugate was lyophilized and stored at 4°C until use.

3.2 Analysis of conjugate

The degree of substitution of the MDI conjugates were assessed using a method based upon the determination of free amino groups by reaction with 2,4,6-trinitrobenzene sulphonic acid (TNBS) as described previously (Rattray *et al.*, 1994). Approximately 1mg of MDI-albumin conjugate and 1mg of albumin were each dissolved in approximately 1ml of 0.1M sodium borate buffer (pH9.3). 25µl of a stock solution of 0.03M TNBS in 0.1M sodium borate buffer (pH9.3) was added to each sample and the samples incubated for approximately 20 minutes at room temperature. The optical density at 420nm (OD) was measured using a Philips spectrophotometer (PU 8880uv/vis). Albumin has approximately 30 readily available hapten-binding sites per molecule. The degree of substitution (moles hapten/moles protein) is calculated according to the formula:-

$$\text{Substitution ratio} = \frac{1 - (\text{OD sample}) \times 30}{(\text{OD albumin})}$$

$$\text{i) RSA-MDI substitution ratio} \quad \frac{1 - (1.207) \times 30}{(1.64)} = 7.92$$

The conjugate substitution ratio was therefore approximately 8:1 moles hapten: moles protein

$$\text{ii) GSA-MDI substitution ratio} \quad \frac{1 - (0.401) \times 30}{(1.699)} = 22.9$$

The conjugate substitution ratio was therefore approximately 23:1 moles hapten: moles protein.

$$\text{iii) HSA-MDI substitution ratio} \quad \frac{1 - (1.099) \times 30}{(1.588)} = 9.23$$

The conjugate substitution ratio was therefore approximately 9:1 moles hapten: moles protein.

3.3 Storage of serum samples

Serum samples were shipped frozen from BASF and arrived in good condition. On arrival, the samples were stored at -70°C until analysis which was completed by 7th October 1999. On first use of the samples, each was aliquotted and stored at -70°C to avoid repeat freeze thawing cycles.

3.4 Enzyme-linked immunosorbent assay (ELISA)

3.4.1 Guinea Pig anti-MDI IgG ELISA

Plastic microtitre plates (NUNC Immunoplate type II, Nunc, Copenhagen, Denmark) were coated with 100µl per well of 25µg/ml MDI-albumin conjugate in 0.5M sodium carbonate/bicarbonate buffer (pH9.6) by overnight incubation at 4°C. This was administered

on to the plate using a 12 channel adjustable repeat dispenser set to take up and dispense 100µl of fluid. The plates were flicked out and washed three times for approximately 3 minutes each wash with 100µl PBS containing 0.05% Tween 20 (PBS-Tween). Guinea pig serum samples were serially diluted in PBS-Tween. The serially diluted guinea pig serum samples were added to duplicate wells (100µl per well). Each plate also had a row of wells to which diluent (PBS-Tween) only was added (reagent blank wells). The plates were incubated for 30 minutes at 37°C. The plates were washed as before and 100µl rabbit anti-guinea pig IgG1 (ICN Biomedicals, Basingstoke, UK), diluted 1 in 1250 in PBS-Tween, was added to each well. After a further 30 minutes incubation at 37°C, plates were washed and 100µl peroxidase-labelled goat anti-rabbit IgG (Harlan Serolab, Crawley Down, UK), diluted 1 in 2500 in PBS-Tween was added to each well. Following 30 minutes incubation at 37°C, the plates were washed and 100µl substrate added (1.6mg/ml O-phenylene diamine and 0.4mg/ml urea hydrogen peroxide in 0.5M citrate phosphate buffer [pH5.0] per well). The reaction was terminated after approximately 10 minutes by the addition of 50µl 0.5M citric acid per well. Absorbance was measured at 450nm using an automated plate reader (Multiskan, Flow Laboratories, Irvine, Ayrshire, UK).

Serum samples were analyzed by ELISA using serial doubling dilutions of 1 in 10 to 1 in 5120.

3.4.2 Rat anti-MDI IgG ELISA Method

Plastic microtitre plates were coated with 100µl per well of 25µg/ml MDI-HSA conjugate or HSA in 0.5M sodium carbonate/bicarbonate buffer (pH9.6) by overnight incubation at 4°C. This was administered on to the plate using a 12 channel adjustable repeat dispenser set to take up and dispense 100µl of fluid. The plates were flicked out and a blocking solution of 5% Marvel/PBS was added to all wells. The plates were incubated at 37°C for 30 minutes. After this period the plates were washed three times for approximately 3 minutes each wash with 100µl PBS containing 0.05% Tween 20 (PBS-Tween). Rat serum samples were serially diluted in PBS-Tween. The serially diluted rat serum samples were added to duplicate wells (50µl per well). Each plate also had a row of wells to which diluent (PBS-Tween) only was added (reagent blank wells). The plates were incubated for approximately 6 hours at 4°C. The plates were washed as before and 100µl goat anti-rat IgG (Serotec, UK), diluted 1 in 1000 in PBS-Tween, was added to each well. The plates were then incubated overnight at 4°C. The

following morning the plates were washed and 100µl peroxidase-labelled donkey anti-sheep/goat (Serotec, UK) diluted 1 in 1000 in PBS-Tween was added to each well. Following a 3 hour incubation at 4°C, the plates were washed and 100µl substrate added (1.6mg/ml O-phenylene diamine and 0.4mg/ml urea hydrogen peroxide in 0.5M citrate phosphate buffer [pH5.0] per well). The reaction was terminated after approximately 10 minutes by the addition of 50µl 0.5M citric acid per well. Absorbance was measured at 450nm using an automated plate reader (Multiskan, Flow Laboratories, Irvine, Ayrshire, UK). Serum samples were analyzed by ELISA using serial doubling dilutions of 1 in 5 to 1 in 1280. All samples were analyzed concurrently against both the MDI-HSA conjugate and HSA as the coating agent.

3.5 Statistical analysis

For each ELISA dilution curve for each individual serum sample run against HSA and MDI-HSA conjugate, the area under the curve was calculated. The areas were considered using analysis of variance followed by log transformation to stabilize the variance. Least-squares means for each group were calculated. Unbiased estimates of differences from concurrent control groups were provided by the difference between each treatment group least-squares mean using MDI-HSA conjugate as substrate with the same serum samples using HSA alone as substrate. Differences from controls were tested statistically by comparison of each group least-squares mean using MDI-HSA as substrate with the same serum samples using HSA alone as substrate using a two-sided student's t test based upon the error mean square in the analysis (SAS, 1996). Differences were considered to be statistically significant at the 5% and 1% level.

4. RESULTS

4.1 ELISA development

Initial experiments using the pooled positive control sera from the rats exposed to MDI intradermally revealed a high level of cross reactivity with the reagents used in the ELISA for detection of rat IgG antibody and RSA (Figure 1). Such cross reactivity was independent of the presence of rat serum samples, with a high level of substrate conversion observed in the reagent blank wells (OD 450nm 1.479), indicating a high degree of cross reactivity with the

RSA and the secondary antibody reagents, presumably due to the presence of contaminating rat immunoglobulins in the commercial preparation of RSA.

The problem of cross reactivity with RSA was exclusive to rat ELISA reagents. Thus the reagents used in the standard guinea pig ELISA methodology (for example in CTL/L/8246) did not cross react with RSA substrate (Figure 2), with low levels of binding detected either in the presence of normal guinea pig serum or in the presence of serum derived from an animal exposed by inhalation to MDI (fifteen times 6 hour exposures to 10mg/m^3 MDI; 153-EU-MTX; CTL/L/8246). The MDI-RSA conjugate prepared for use in the current study was a good substrate for guinea pig specific anti-MDI antibody detection. Thus normal guinea pig serum failed to display any reactivity whereas good binding was observed for the serum derived from the MDI exposed animal. Indeed, this specific binding was equivalent to that observed using a standard MDI-GSA conjugate (Figure 2), despite the fact that the MDI-RSA conjugate had a somewhat lower substitution ratio than the MDI-GSA conjugate (8:1 compared with 22:1, respectively).

Given that RSA was clearly an inappropriate carrier protein for preparation of a conjugate for analysis of rat antibody responses, the suitability of albumins from other species for conjugate production was investigated. Figure 3 displays background binding of pooled rat serum samples derived from animals exposed intradermally to MDI (-1, 7, 14 and 21 days after initiation of exposure) against bovine serum albumin (BSA), GSA and HSA. Unacceptably high levels of background binding were observed for BSA and GSA substrates, particularly with samples isolated 14 and 21 days after initiation of exposure. Relatively low levels of nonspecific binding were detected when HSA was used as substrate, with exposure to MDI having no effect on background binding.

HSA was therefore chosen as the most suitable carrier protein for conjugate preparation for the development of the rat anti-MDI specific ELISA. In subsequent experiments negative control sera (pooled from animals bled prior to exposure) and the positive control sera (pooled from rats 21 days after intradermal exposure to MDI) were used with the MDI-HSA conjugate in comparison with activity against HSA alone to optimize for the detection of MDI-specific IgG antibody. Figure 4a and b display the detection of specific antibody using

the standard ELISA conditions as utilized for the detection of guinea pig anti-MDI IgG antibody. Although nonspecific binding to HSA was very low for both positive and negative control sera (Figure 4a), the degree of specific binding of positive control sera to MDI-HSA conjugate was relatively low compared with what would be expected in for example sera derived from guinea pigs immunized under similar conditions (Figure 4b). Various parameters were therefore changed (including increased incubation times, incubation temperature and blocking buffers) in order to optimize specific binding to the MDI-HSA conjugate. Figure 4c and d show results obtained for the final ELISA protocol (as described in the Materials and Methods section of this report). Some nonspecific binding to HSA alone was observed for both negative control and positive control sera (Figure 4c) which was largely due to the relatively high serum concentration applied (1 in 5 starting dilution). For the negative control sera, equivalent levels of background binding were seen irrespective of the presence of MDI hapten (HSA versus MDI-HSA conjugate; Figure 4c versus d). However, positive control sera showed a marked degree of specific binding, with substantially higher OD450nm values obtained in the presence of MDI hapten (HSA versus MDI-HSA conjugate; Figure 4c versus d).

4.2 Serum analyses

In subsequent analyses, pooled serum samples from isolated from animals -1, 7, 14 and 21 days after intradermal exposure to MDI were analyzed concurrently against HSA alone and MDI-HSA conjugate (Figure 5a and b). All samples displayed some reactivity against HSA alone, although this background binding was unaffected by exposure to MDI (Figure 5a). Serum samples isolated after MDI exposure displayed a time dependent increase of specific anti-MDI IgG production, reaching maximal levels 14 to 21 days after initiation of exposure to MDI. However, intradermal exposure to MDI did not result in the production of high titre antibody, with all serum samples displaying titres of less than 1 in 320 (OD450nm readings of serum from MDI-treated animals were identical to naïve rat serum at this dilution).

Pooled serum samples derived from animals at various times (days -1, 7, 14 or 21) after topical exposure to MDI, with or without a skin wash at 8 hours, were analyzed concurrently against HSA alone and MDI-HSA conjugate (Figure 6a, b, c and d). As reported for serum samples derived from intradermally exposed animals (cf Figure 5), all samples displayed

some reactivity against HSA alone. This background binding was unaffected by exposure to MDI (Figure 6a and c), indeed, the highest levels of background binding were observed generally in samples isolated prior to treatment with MDI. Only serum samples isolated after topical application of MDI displayed detectable specific anti-MDI IgG antibody, reaching maximal levels 14 to 21 days after initiation of exposure to MDI. The time course and the antibody dilution profile of anti-MDI-IgG antibody were very similar whether rats had been exposed topically to MDI without a skin wash (Figure 6a and b) or whether they had received a wash 8 hours after application of MDI (Figure 6c and d). Indeed, given the short biological half-life of this molecule in biological fluid, a skin wash after 8 hours would not be expected to have a substantial effect on antibody responses. In both cases, anti-MDI IgG antibody production was also very similar to that induced by intradermal exposure to MDI (cf Figure 5a and b), with all treatments resulting in the production of relatively low titre antibody, with all serum samples displaying titres of less than 1 in 320 (OD450nm readings of serum from MDI-treated animals were identical to naïve rat serum at this dilution).

Subsequently, individual serum samples from animals exposed topically to MDI, with and without a skin wash at 8 hours, were analyzed by ELISA for anti-MDI IgG antibody content against HSA alone and MDI-HSA conjugate concurrently. The results from rats exposed dermally to MDI without a skin wash are displayed in Figure 7, and those for animals which received a skin wash at 8 hours are shown in Figure 8. There was very little interanimal variation in the relatively low levels of background binding to HSA in serum from the five animals which were exposed to MDI without any washing procedure (Figure 7a; day -1). These day -1 samples displayed no specific anti-MDI IgG antibody activity, with low level binding observed when MDI-HSA was used as substrate (Figure 7b). As observed for the pooled serum samples, there was no increase in background binding to HSA following exposure to MDI (Figure 7c, e, and f; day 7, 14 and 21 respectively). Exposure to MDI resulted in the production of specific anti-MDI IgG antibody in all five animals, with maximal responses recorded 14 to 21 days after initiation of exposure (Figure 7d, f and h). A similar pattern was observed for serum samples derived from animals which received a skin wash 8 hours after topical treatment (Figure 8), with the exception of one individual animal which displayed relatively high background activity against HSA substrate, particularly on day 7 and 14, and another individual animal which displayed relatively high activity against

MDI-HSA substrate on day -1. This larger degree of interanimal variation reflects presumably the increased group size for this treatment group ($n = 10$). Notwithstanding the increased variability, as observed for the pooled serum samples, exposure topically to MDI with a skin wash at 8 hours resulted in the appearance of detectable anti-MDI IgG antibody with maximal levels recorded at 14 and 21 days. All treatments resulted in the production of relatively low titre antibody, with all serum samples displaying titres of less than 1 in 320 (OD450nm readings of serum from MDI-treated animals were identical to naïve rat serum at this dilution).

For each topical treatment group at each time point, the statistical significance of anti-MDI IgG antibody responses was assessed by comparison of serum reactivity against MDI-HSA conjugate against HSA substrate alone (Table 1). These analyses revealed that topical treatment with MDI, with or without a skin wash at 8 hours, resulted in significant antibody responses ($p = 0.05$; $p < 0.01$) 14 and 21 days after initiation of exposure.

5. CONCLUSIONS

An ELISA assay has been developed for the analysis of rat anti-MDI IgG antibody responses. The ELISA has been developed using a conjugate prepared with HSA and MDI monomer, as rat IgG displayed the lowest levels of non-specific binding to HSA compared with all of the other substrates tested.

Pooled serum samples derived from animals exposed intradermally to MDI (provided by BASF as a positive control for the ELISA development) were analyzed by ELISA for IgG antibody responses. Activity against MDI-HSA conjugate was compared against activity against HSA alone as substrate to determine specific anti-MDI antibody responses. There was no specific antibody detected in serum derived from animals on day -1 before exposure, but there was a time-dependent increase in specific anti-MDI IgG antibody following intradermal treatment with MDI, with substantial IgG anti-MDI activity observed in sera isolated 7, 14 and 21 days after intradermal exposure to MDI. The rat anti-MDI IgG antibody responses titred out relatively quickly, with no specific binding (equivalent levels of IgG

binding to HSA substrate alone compared with binding to MDI-HSA conjugate) observed at dilutions of 1 in 320 or less.

Exposure of rats to MDI by topical exposure elicited significant anti-MDI IgG antibody production. Detectable antibody was observed first at 7 days after initiation of exposure and reached maximal levels after 14 and 21 days. Similar kinetics and magnitude of antibody responses were observed regardless of whether the animals received a skin wash 8 hours after treatment, indicating that the animals received most of the immunogenic dose of MDI within the first 8 hours of exposure, although the degree of specific binding was relatively low. A further consideration is that due to the short biological half-life of this molecule in biological fluid, a skin wash after 8 hours would not be expected to have a substantial effect on antibody responses. Without exception, relatively low titre antibody responses were observed, with no specific MDI IgG antibody detected at dilutions of 1 in 320 and below. Such titres are, however, similar to those observed following topical application of MDI to guinea pigs, with a single topical application of 30% MDI provoking detectable antibody responses in 5 out of 8 animals, with titres ranging from 1 in 40 to 1 in 2560 (Rattray *et al.*, 1994).

Thus it would appear from this experiment that in the rat both intradermal injection and topical exposure to MDI induce significant, but relatively low, levels of anti-MDI IgG antibody. In the guinea pig, intradermal injection of MDI is very effective at stimulating a marked high titre IgG antibody response whereas topical exposure results generally in low titre antibody. This preliminary evidence suggests that there are inter-species variations in immunological responsiveness to MDI, however it is not possible to determine from these experiments whether the route of exposure is the important variable or whether this strain of rat is inherently less responsive to MDI regardless of the route of exposure employed. It must also be noted that the experimental protocol for the topical exposure of rats to MDI was not identical to that utilized previously for topical exposure of guinea pigs to MDI; such differences in experimental design may contribute to the observed differences in antibody expression. It should also be noted that there are other differences in experimental design which may contribute to the apparent species differences, including the use of homologous versus heterologous carrier proteins for conjugate preparation, the antigenicity of the conjugates and the influence of hapten substitution ratios on the detection of antibody.

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NJ Rattray, PA Botham, PM Hext, DR Woodcock, I Feilding, RJ Dearman and I Kimber (1994). Induction of respiratory hypersensitivity to diphenylmethane-4,4-diisocyanate (MDI) in guinea pigs. Influence of route of exposure. *Toxicology*, **88**: 15-30

CTL/R/1211 Evaluation of antibody responses in guinea pigs following intradermal induction and MDI challenge.

CTL/L/8246 153-EU-MTX : Guinea pig model for MDI asthma.

SAS Institute Inc. SAS/STAT User's Guide, Version 6, Fourth Edition, Volume 2, Cary, NC : SAS Institute Inc., 1989.

TABLE 1 - STATISTICAL ANALYSIS OF ANTI-MDI IgG ANTIBODY RESPONSES

A. Dermal exposure without skin wash

Day	Mean area under curve		SD	P value	Significance
-1	HSA	113.403	5.347	ND	ND
	MDI-HSA	106.233	5.859		
7	HSA	132.043	3.382	0.539	NS
	MDI-HSA	139.233	19.821		
14	HSA	147.036	10.658	0.029	p = 0.05
	MDI-HSA	261.941	77.062		
21	HSA	156.768	18.671	0.034	p = 0.05
	MDI-HSA				

B. Dermal exposure with skin wash at 8 hours

Day		Mean area under curve	SD	P value	Significance
-1	HSA	149.99	5.448	0.850	NS
	MDI-HSA	151.619	30.346		
7	HSA	205.166	23.336	0.190	NS
	MDI-HSA	220.204	44.053		
14	HSA	175.581	16.004	<0.001	p < 0.01
	MDI-HSA	245.002	43.182		
21	HSA	140.143	12.607	<0.001	p < 0.01
	MDI-HSA	236.952	34.585		

ND = not determined

NS = not significant

FIGURE LEGENDS AND FIGURES

Figure 1 Binding of rat ELISA reagents to rat serum albumin (RSA)

Binding of rat ELISA reagents to RSA in the presence or absence of rat serum samples. Concurrent reagent blank (binding in the absence of rat serum) value (●) is shown with OD450nm values obtained with various dilutions of pooled rat serum samples derived from animals exposed intradermally to MDI and isolated -1 (◆), 7 (■), 14 (▲) and 21 (✕) days following initiation of exposure.

Figure 2 Profiles of antibody responses of guinea pig serum samples from study 153-EU-MTX) : a comparison of MDI-RSA substrate with MDI-GSA substrate

Profiles of antibody responses following inhalation exposure of guinea pigs to MDI (fifteen times 6 hour exposures to 10mg/m^3) using a standard MDI-GSA substrate, MDI-RSA substrate or RSA alone. Serum samples from MDI exposed animals (closed symbols) or normal guinea pig serum (open symbols) were analyzed in a standard guinea pig anti-MDI IgG ELISA using RSA (●), MDI-RSA (■) or MDI-GSA (▲) as substrates. Concurrent reagent blank (binding in the absence of rat serum) value is shown as (●).

Figure 3 Background binding of rat serum samples to BSA, GSA or HSA substrates.

Background binding of pooled serum samples isolated from animals exposed intradermally to MDI day -1 (◆), day 7 (■), day 14 (▲) or day 21 (✕) after initiation of exposure against BSA (a), GSA (b) or HSA (c) substrates. Concurrent reagent blank (binding in the absence of rat serum) value is shown as (●).

Figure 4 Antibody response profiles following intradermal exposure of rats to MDI : a comparison of ELISA methods

Profiles of antibody responses following intradermal exposure of rats to MDI. Pooled serum samples from day -1 (◆) and day 21 (✕) following initiation of intradermal exposure to MDI

are shown using an ELISA protocol analogous to the method used for detection of guinea pig anti-MDI IgG antibody (a, b) or using the optimized protocol for detection of rat anti-MDI IgG antibody as described in the Materials and Methods section (c, d). In each case, serum samples were run against HSA alone (a, c) or against MDI-HSA substrate (b, d).

Concurrent reagent blank (binding in the absence of rat serum) value is shown as (●).

Figure 5 Profiles of antibody responses following intradermal exposure of rats to MDI. Profiles of antibody responses in pooled serum samples isolated on day -1 (●), day 7 (■), day 14 (▲) or day 21 (×) following intradermal exposure to MDI. Serum samples were run in the standard rat anti-MDI IgG ELISA as described in the Materials and Methods section using HSA substrate (a) or MDI-HSA substrate (b). Concurrent reagent blank (binding in the absence of rat serum) value is shown as (●).

Figure 6 Profiles of antibody responses following topical exposure of rats to MDI. Profiles of antibody responses in pooled serum samples isolated on day -1 (●), day 7 (■), day 14 (▲) or day 21 (×) following topical exposure to MDI without a skin wash (a, b) and with a skin wash at 8 hours (c, d). Serum samples were run in the standard rat anti-MDI IgG ELISA as described in the Materials and Methods section using HSA substrate (a, c) or MDI-HSA substrate (b, d). Concurrent reagent blank (binding in the absence of rat serum) value is shown as (●).

Figure 7 Profiles of antibody responses following topical exposure of rats to MDI. Profiles of antibody responses in individual serum samples isolated on day -1 (a, b), day 7 (c, d), day 14 (e, f) or day 21 (g, h) following topical exposure to MDI without a skin wash. Serum samples were run in the standard rat anti-MDI IgG ELISA as described in the Materials and Methods section using HSA substrate (a, c, e, g) or MDI-HSA substrate (b, d, f, h). Each individual serum sample is represented by a single line. Concurrent reagent blank (binding in the absence of rat serum) value is shown as (●).

Figure 8 Profiles of antibody responses following topical exposure of rats to MDI. Profiles of antibody responses in individual serum samples isolated on day -1 (a, b), day 7

(c, d), day 14 (e, f) or day 21 (g, h) following topical exposure to MDI with a skin wash at 8 hours. Serum samples were run in the standard rat anti-MDI IgG ELISA as described in the Materials and Methods section using HSA substrate (a, c, e, g) or MDI-HSA substrate (b, d, f, h). Each individual serum sample is represented by a single line. Concurrent reagent blank (binding in the absence of rat serum) value is shown as (●).

Figure 1

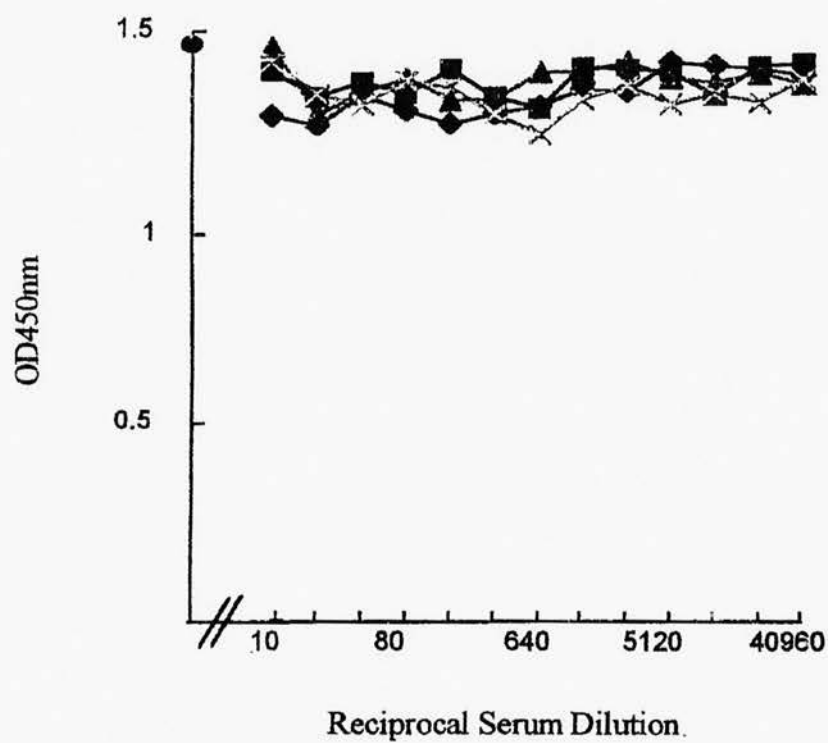


Figure 2

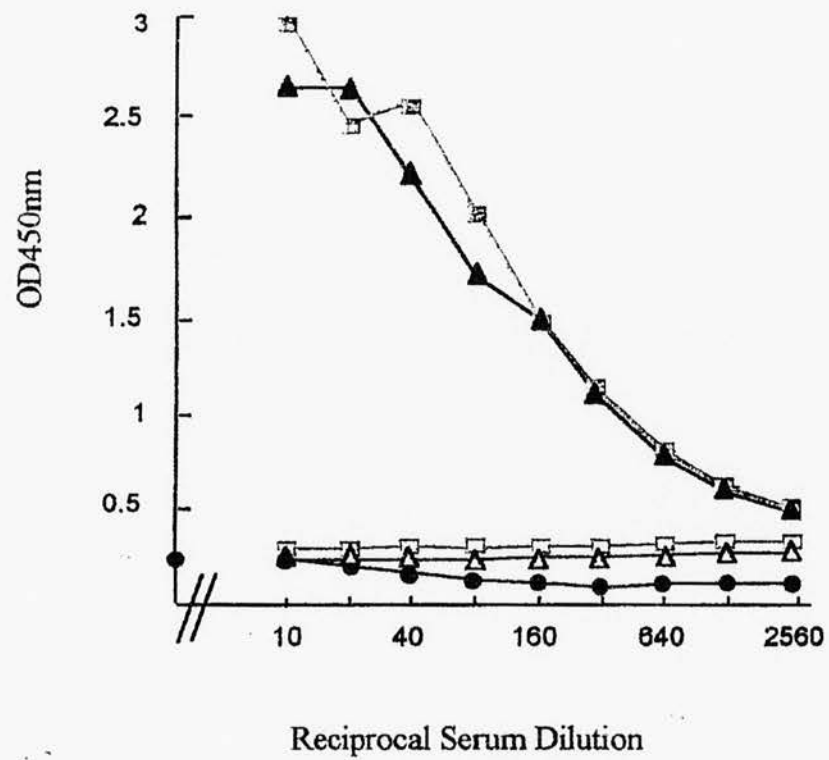


FIGURE 3

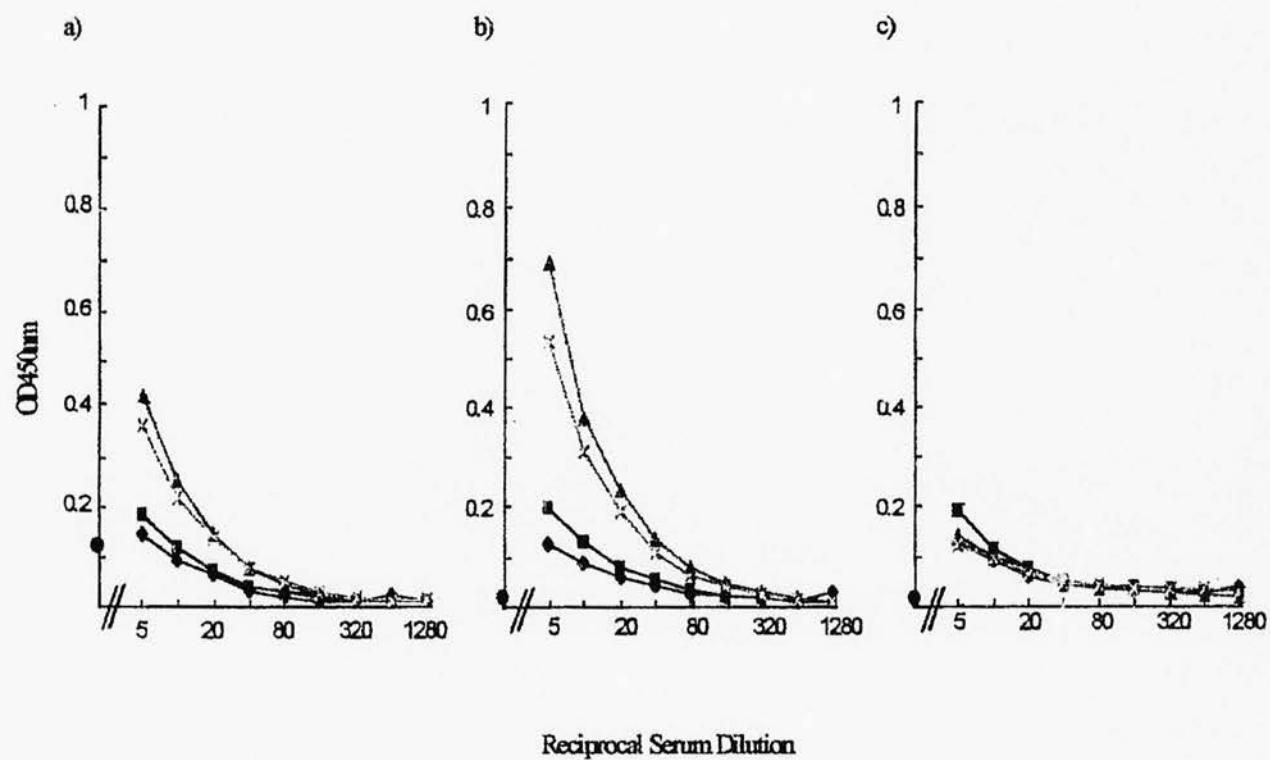


Figure 4

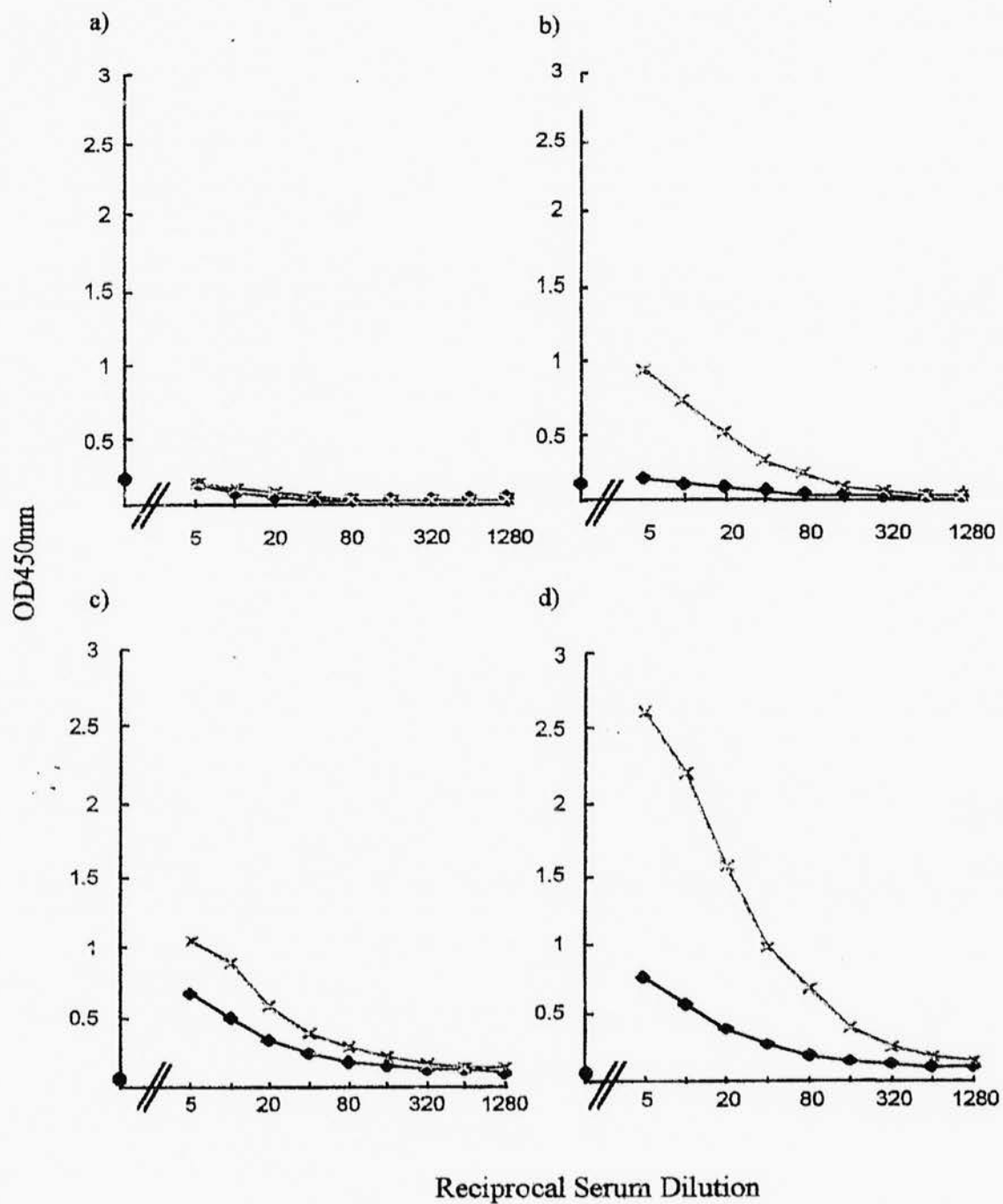


FIGURE 5

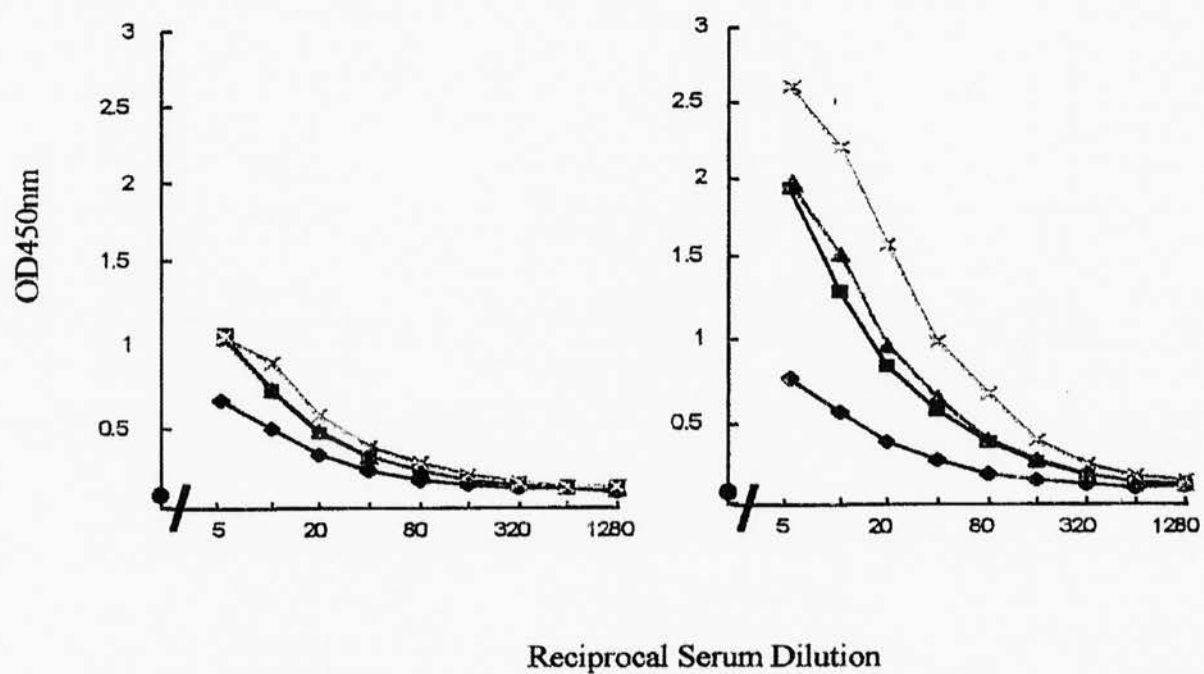


FIGURE 6

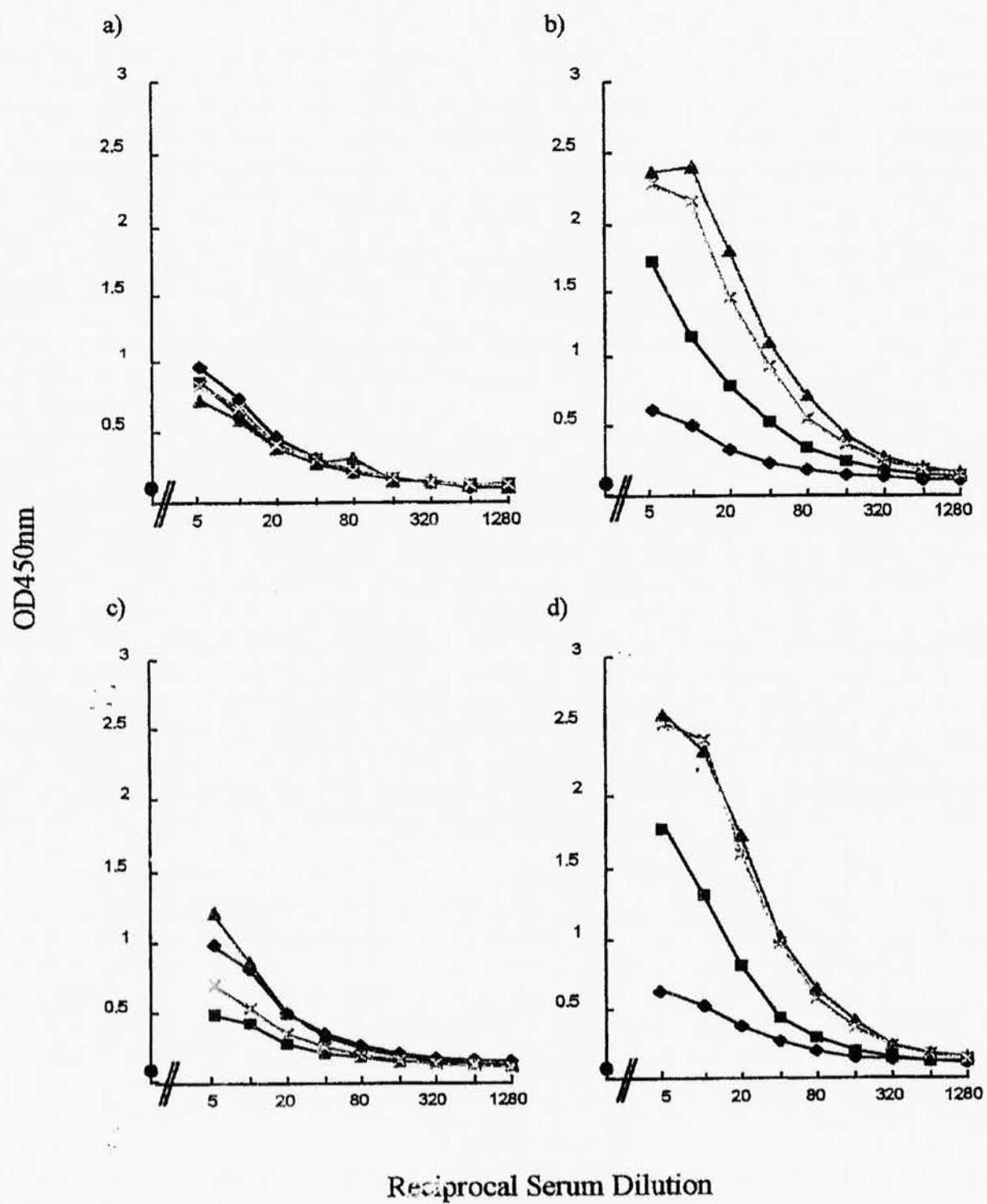


FIGURE 7

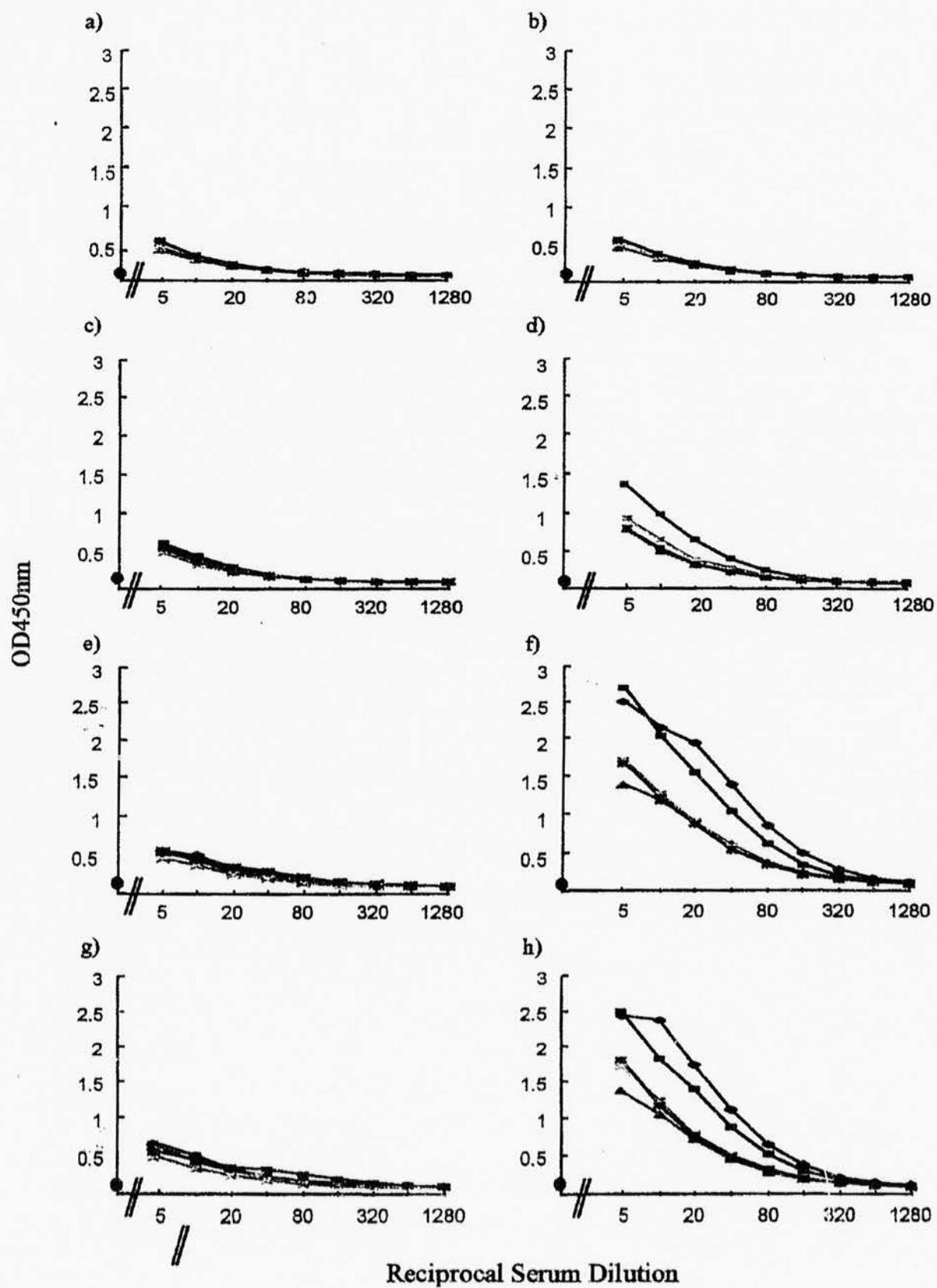
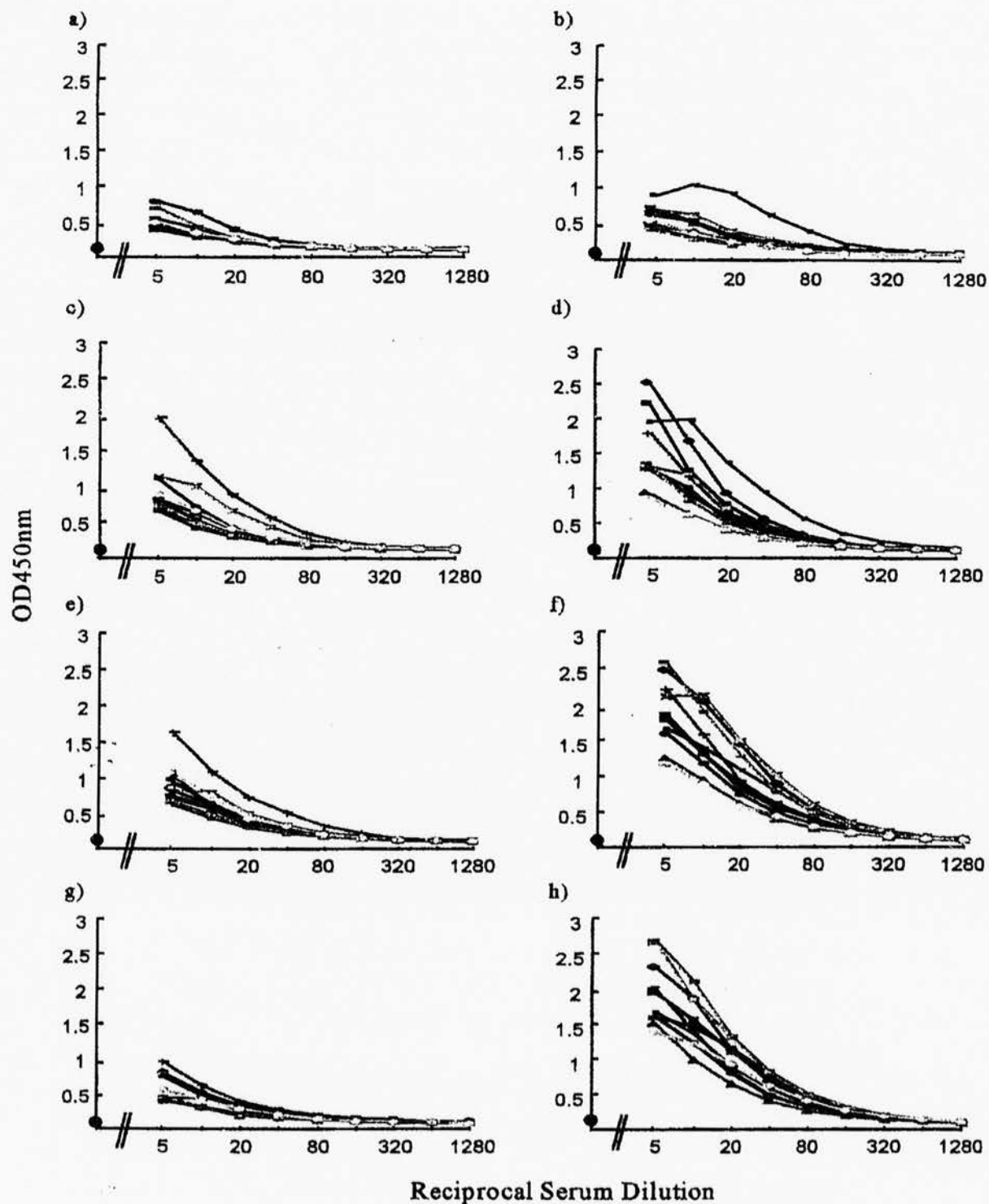


FIGURE 8



CERTIFICATE OF AUTHENTICITY

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